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IMMUNOLOGICAL DETERMINATION OF CHEMOKINE'S ASSOCIATED WITH ISCHEMIC HEART DISEASE

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Summary
The aim of present study is to shed the light on the impact chemokine's CCL2, CCL5 and CXCL9 serum level associated with ischemic heart disease IHD of susceptibility and development. The patients were divided according to the clinical condition into three types (stable angina pectoris SAP, unstable angina pectoris UAP, and MI). Which was diagnosed by cardiologists and blood vessels. In this study, the chemokine's were included : measurement of (CCL2, CCL5 and CXCL9) by ELISA. in present study showed that that the mean of serum levels of all chemokine's (pg\ml) among ischemic heart disease IHD patients was higher than control group: where CCL2 was have [(135, 56 $\pm$ 51.48) and (18, 28 $\pm$ 17, 24)] respectively, CCL5 was have [(19.77 $\pm$ 5.81) and (5.93 $\pm$ 1.70)] respectively, and CXCL9 that have [(268.354 $\pm$ 249.981) and (25.554 $\pm$ 19.294)] respectively. statistically the differences was highly significant between IHD and control group that have (P-value 0.001) for all chemokine's study. In present study, when classifying patients group with ischemic heart disease into three types (SAP, UAP and MI), it was noted that the mean levels of CCL2 chemokine in the serum were uneven between the three types, as they were CCL2 serum level is higher at SAP than UAP and MI, compared with the control group. statistically the differences was very highly significant for SAP and significant with UAP and no significant with MI that have [(P-value 0.001), (P-value 0.011) and (P-value 0.302)] respectively. In this study, it was noted that the mean levels of CCL5 chemokine in UAP was slightly high than SAP and MI but, all the results showed an increase in CCL5 serum levels when compared with the control group. statistically the differences was very highly significant for SAP and and no significant with UAP and MI that have [(P-value 0.001), (P-value 0.236) and (P-value 0.103)] respectively. While regarded CXCL9 chemokine, this study noted the mean levels of CXCL9 chemokine in serum of MI was slightly high than SAP and high than USP but, all the results showed an increase of CXCL9 serum levels when compared with the control group. statistically the differences was very highly significant for SAP and and no significant with UAP and MI that have [(P-value 0.001), (P-value 0.494) and (P-value 0.279)] respectively. In this present study, when samples were taken from the vein and artery of patient with IHD, and comparison the results between them. It was found that the mean level of chemokine (CCL2, CCL5 and CXCL9) in artery sample was higher than mean level of vein sample. statistically [(P-value 0.016), (P-value 0.005) and (P-value 0.0.262)] respectively.

Key words: Ischemic heart disease IHD, atherosclerosis, Chemokine's, CCL2, CCL5, CXCL9.

Introduction

Cardiovascular disease CVD is the leading cause of death worldwide. The number of cardiovascular cases of death is higher than cancer and more than any other cause of death (**Benjamin, *et al*., 2019**). The most prevalent type of CVDs and death is ischemic heart disease (IHD) (**khan, *et al*., 2020**)

Ischemic heart disease (IHD) is often referred to as coronary artery disease (CAD) , atherosclerotic cardiovascular disease (ACD) and coronary heart disease (CHD), is a condition in which there is an inadequate supply of blood and oxygen to aportion of the myocardium. The highest prevalence rates of IHD are seen in Eastern Europe, North Africa, and the Middle East (**Virani, *et al*., 2020**). According to WHO data published in 2018, IHD deaths in Iraq reached 32, 463 or 18, 92% of total deaths (**Buchari, 2018**).

The primary pathological process that leads to IHD is atherosclerosis, an inflammatory disease of the arteries associated with lipid deposition and metabolic alterations due to multiple risk factors. More than 70 % of at - risk individuals have multiple risk factors for IHD, and only 2 % -7 % of the general population have no risk factors (**Sampasa, *et al*., 2015**). Atherosclerosis is a chronic inflammatory disease, with immune cells and their effector molecules initiating and maintaining the progression of atherosclerotic lesion formation, (**szentes, *et al*., 2018**). Atherosclerosis is a disease caused by the development of layers of fatty deposits on the inner walls and accumulation of immune cells in site of the arteries (**Amirfakhryan, 2019**).

Chemokine's are a family of chemoattractant cytokines (small proteins secreted by cells that influence the immune system) which play a vital role in cell migration through venules from blood into tissue and vice versa, and in the induction of cell movement in response to a chemical (chemokine) gradient by a process known as chemotaxis. (**Mamazhakypov, *et al*., 2019**). Chemokine's constitute a family of small (8-14 kDa) secreted peptides that bind and activate chemokine receptors. So far 53- human chemokines and 23-chemokine receptors have been identified (**Márquez, *et al*., 2021**). Chemokine's have an important role in the development and progression of ischemic heart disease IHD and formation of plague in the blood vessels (**Lu, et al., 2021**) chemokine's cause an increase in the recruitment of immune cells and their accumulation in sites of chronic inflammation, especially in patients with atherosclerosis, which leads to ischemic heart disease.(**Marquez, *et al*, .2021** and **Noels, *et al*, .2019**). CCL2 Chemokine : [Monocyte Chemoattractant Protein-1(MCP-1)], is a potent-inflammatory chemokine and signals through its receptor CCR2 (**Deshmane, et al., 2009**) . CCL2 can be secreted by many cells including monocytes ,smooth muscle cells endothelial cells, and platelets (**Liu-Dan, *et al*., 2015**). this chemokine's have great attention in the research of atherosclerosis as they play a key role in development and progression of atherosclerotic lesions and stable plaque that causesischemic heart disease IHD . They activation, recruitment, and infiltration of immune cells (**Lu, *et al*., 2021** and **Gencer, *et al*., 2021** and **Andersen, *et al*., 2019)**. CCL5 Chemokine: also known as RANTES (regulated on activation, produced and released by normal T-cells) (**Appay, *et al*., 2001**). CCL5 mediates the chemotaxis and activation of T cells, monocytes, granulocytes, mast cells, and dendritic cells (**von, *et al*., 2001**). CCL5 is reported to bind to numerous receptors, including CCR1, CCR3, and CCR5. (**Marques, *et al*., 2013**). The chemokine CXCL9, also known as Monokine induced by gamma (MIG), is produced in response to IFN- and may serve to amplify the IFN- signal. Several researchers confirm this. CXCL9, like CXCL10 and CXCL11, is an inflammatory chemokine that binds to the CXCR3 receptor. They primarily direct the immunological actions of activated T cells. (**Tokunaga, *et al*., 2018**).

Materials and methods

cases-control study was collection of blood samples and experimental protocols, were approved by the scientific and ethical committee in college of medicine–university of Basrah and the research ethics committees of AL-Imam Sadiq Teaching Hospital, Babel Specialized Center for Catheterization and Cardiac Surgery, and the University of Al-Basrah.

Seventy patients with ischemic heart disease IHD and 80 healthy controls will be included in the research. Patients and controls. Blood samples from patient and from the controls (Patients and controls should be compatible by age group and sex). withdraw 5ml of blood and then placed 3ml in a gel tube and left for 10 minutes to coagulate, then it will be separated in the centrifuge to obtain the serum sample to detection levels of chemokine's. Using the ELISA method (quantitative sandwich ELISA method) to determine the amount of of chemokine's (CCL2, CCL5 and CXCL9), in serum of patients with ischemic heart disease IHD .

Statistical analysis

All statistical analyzes were performed using Statistical Package of Social Sciences SPSS (ver-24) . Kruskal-Wallis Test and Mann Whitney U Test were used to find the correlation between the quantitative variables. The level of significance was set at ($P < 0.05$).

Results:

Differences in the level of chemokine's (CCL2, CCL5 and CXCL9) between patient (IHD) and controls.

In Table (1) showed of patients and controls significantly statistically differed in the levels of the studied chemokine's, when the levels were higher in patients than in controls, For all of chemokine's, (p. value=0.0001) .

Table (1): Comparison between patient (IHD) and control groups according to chemokine's CCL2, CCL5 and CXCL9 .

Cases		**CCL2 (pg/ml)**	**CCL5 (pg/ml)**	**CXCL9 (pg/ml)**
Patient	N	70	70	70
	Mean	135.56418	19.77666	268.35425
	SD	151.48393	5.810094	249.98103
Control	N	80	80	80
	Mean	18.285994	5.934217	25.554311
	SD	17.247557	1.707181	19.294732
Sig.*		0.0001	0.0001	0.0001

* Mann Whitney U Test

Differences in the level of chemokine's (CCL2, CCL5 and CXCL9) between patient sub-groups (MI, UAP and SAP) and controls group.

When the levels of the investigated chemokine's were compared according to the type of the IHD, it was found that all the chemokine's levels where significantly statistically higher in angina pectoris patients than controls ($p< 0.0001$ in all chemokine's study, Table (2). Binary differences were tested and significant statistical differences were found.

Table (2): Comparison among control group and patient sub-groups (SAP UAP and MI) according to CCL2, CCL5 and CXCL9.

Cases		**CCL2 (pg/ml)**	**CCL5 (pg/ml)**	**CXCL9 (pg/ml)**
Control	N	80	80	80
	Mean	18.285994	5.934217	25.554311
	SD	17.2475579	1.7071818	19.2947327
MI	N	34	34	34
	Mean	112.679688	19.067285	285.558824
	SD	160.1671001	6.0698937	235.5396309

UAP	N	16	16	16
	Mean	132.221081	21.581325	216.976181
	SD	173.3089507	5.0394539	257.9327441
SAP	N	20	20	20
	Mean	177.142310	19.538870	280.208945
	SD	110.9064681	5.9032428	274.1486514
Sig.**		0.0001	0.0001	0.0001
Control with MI Sig.*		0.302	0.103	0.279
Control with UAP Sig.*		0.011	0.236	0.494
Control with SAP Sig.*		0.0001	0.0001	0.0001
MI with UAP Sig.*		0.038	0.171	0.308
MI with SAP Sig.*		0.0001	0.0001	0.0001
UAP with SAP Sig.*		0.0001	0.0001	0.0001

* Kruskal-Wallis Test
** Mann Whitney U Test

Relationship level of chemokine's with clinical sample:
Table (3) showed the mean level of CCL2 in artery sample higher when compare with level in vein sample, there is no significant statistical differences between Clinical Samples (Vein / Artery) with CCL2 .

Table (3): Shows the relationship between levels of chemokine's (CCL2, CCL5 and CXCL9) with Clinical sample (IHD).

Clinical sample (IHD)		**CCL2 (pg/ml)**	**CCL5 (pg/ml)**	**CXCL9 (pg/ml)**
Vein	Mean	100.080222	16.220680	211.184401
	S.D	137.0507914	7.7386449	233.9138805
Artery	Mean	212.478144	23.305389	284.578633
	S.D	165.1599156	2.3888399	326.1346396
Sig*		0.016	0.005	0.262

* Mann Whitney U Test

Discussion:
chemokine CCL2 (C-C ligand 2) :
CCL2 as the first member of the CC-chemokine subfamily to be discovered, CCL2 is released by a wide variety of cells, including monocytes, endothelial cells, smooth muscle cells, and platelets (**Liu-Dan, *et al*., 2015)**. It is also known by the acronym MCP-1. Most cardiovascular risk factors, markers of coronary atherosclerosis load, and incident cases of coronary and peripheral artery disease are positively linked with plasma levels of MCP-1 in population studies. (**Herder, *et al*., 2006**).
In the development of atherosclerosis, CCL2 is regarded as a pivotal mediator. The best-studied CC-chemokine, monocyte chemoattractant protein (MCP)-1, was initially discovered to be a monocyte-specific chemo attractant, but subsequent research showed that it also attracted T lymphocytes and natural killer cells but not neutrophils. In certain pathologic situations characterised by inflammation and mononuclear cell infiltration, increased expression of MCP-1 was seen. CCL2 is extensively produced in atherosclerotic plaques and mediates macrophage recruitment in the athermanous lesion, according to numerous experimental findings. (**Gencer, *et al*., 2021**).
Multiple studies have shown that CCL2 and other chemokines are elevated in ischemic heart disease, as discussed by **Serrano and M, .(2003)**. The levels of CCL2 in patients were considerably (P = 0.001) greater than those in the control group.

In the current study, results documented that the mean levels of chemokine's CCL2 (pg\ml) among ischemic heart disease patients was higher than control group that have [(135, 56 ± 51.48) and (18, 28 ± 17, 24)] respectively, Statistically, the differences were highly significant (P-value 0.001) and confirm the findings of the previous study (**Koper, *et al.*, 2019** and **De-Lemos, *et al.*, 2003**). Additionally, they discovered a significant increase of CCL2 (P=0.001) in patients compared to controls, and the levels of CCL2 chemokine were correlated with the severity of the disease.

In present study, when classifying patients with ischemic heart disease into three types (stable Angina pectoris (SAP), Unstable angina pectoris (UAP) and Myocardial infarction (MI)), it was noted that the mean levels of CCL2 chemokine in the serum were uneven between the three types, as they were CCL2 serum level is higher at SAP than UAP and MI [(177, 14 ± 110, 90) and (132.22 ± 173.30) and (112, 67 ± 160, 16)] respectively. All the results showed an increase in CCL2 serum levels when compared with the control group (18.285 ± 17.247). statistically the differences was very highly significant for SAP and significant for UAP and no significant for MI that have [(P-value 0.001), (P-value 0.011) and (P-value 0.302)] respectively. The results have been confirmed by previous studies (**Koper, et al, .2019**).

Koper, et al, . (2019) were mentioned above, Both the MI group and the SAP group had greater CCL2 levels compared to the control group, however the difference was statistically significant only for the SAP group (P = 0.004). statistically significant (P > 0.05) when utilising healthy volunteers.

Our findings contradicted those of **Arakelyan et al. (2005)**, who found significantly higher CCL2 concentrations in the MI group compared to the control group. However, there are some variations between these two studies regarding risk factors: 1- 14.7% of participants in our MI group had hypertension, compared to 44% of individuals in the Arakelyan group; 2- 88.2% of participants in our MI group had diabetes, compared to 18% of participants in the Arakelyan, *et al.*, group. Previous research has linked elevated CCL2 levels to hypertension and diabetes.

On the other hand, When comparing CCL2 levels in AMI patients and healthy controls, we found no differences, which is consistent with the findings of **Murakami, *et al.*, (2003)**. Murakami, *et al*., (2003) discovered an interesting phenomenon: after 7 days, CCL2 levels increased considerably following myocardial infarction. In addition, **Economou, *et al.*, (2001)** found that CCL2 concentrations increased significantly 3 and 6 months after PCTA (PTCA). At the time of the patient's initial hospital stay, we measured the amount of CCL2. So, the timing of sample collection and quantitative examination of CCL2 concentrations for subsequent clinical interpretation is crucial.

In this study arteries, when taken blood samples from artery and vein of the same patient to compare the levels of chemokine in the vein sample and the artery sample, found that patient with ischemic heart disease IHD have increase of mean ± SD chemokine CCL2 serum level in the artery compared to the mean ± SD in the vein [(212.478 ± 165.159) and (100.080 ± 137.050)] respectively. This result may be possible that the Atherosclerosis plague causes extensive harm to the vascular tissue. Damaged endothelium (ECs) becomes activated and initiates an inflammatory response in order to recruit immune cells to the site of injury in response to endothelial damage caused by pathogenic variables such as hyperlipidemia and hemodynamic shear stress.. In the other hand, endothelial cells (ECs) and smooth muscle cell can secrete chemokine's included CCL2 in cite of inflammation. (**Gencer, *et al*, . 2021** and **Lacolley, *et al*, . 2015**).

chemokine CCL5 (C-C ligand 5) :

CCL5 (Chemokine ligand-5) is a key mediator of chemotaxisaand activation not just of T cells (**von, *et al.*, 2001** and **Schall, *et al.*, 1991**); but also of monocytes, ggranulocytes, mast cells, and dendritice cells. Though T cells are the primary source of CCL5, additional key cellular sources include platelets, adipocytes, monocytes/-macrophages, and fibroblasts (**Skurk, *et al.*, 2009**). CCL5 (RANTES) is related with cardiovascular risk factors when it is expressed at higher levels in-adipose tissue and when its serum concentrations are higher (**Huber, *et al.*, 2008** and **Herder, *et al.*, 2005**).

RANTES (Regulated upon Activation, Normal T cell Expressed and presumably Secreted) is a CC-chemokine involved in the pathogenesis of cardiovascular disease that is expressed by several cell

types such as T cells, fibroblasts, and some types of tissue monocytes (**Virani, *et al.*, 2011**). It causes leukocyte chemotaxis onto the endothelium wall and induces leukocyte trans endothelial migration, the two hallmarks of thermogenesis. Furthermore, after platelet activation, CCL5 is released from -granules and deposited on the surface of injured endothelial cells. This chemokine is believed to play an important role in the atherogenic recruitment of monocytes by activated platelets, a process that may accelerate the formation of atherosclerotic plaques. Based on what we know from **S-Koh, *et al*., (2009)**.

In the current study, results documented that the mean levels of chemokine's CCL5 (pg\ml) among ischemic heart disease patients was higher than control group that have [(19.77 $\pm$ 5.81) and (5.93 $\pm$ 1.70)] respectively, statistically the differences was highly-significant that have (P-value 0.001) and confirm the results reported by the previous study (**Canouï, *et al*, .2011**) that found significant increase of CCL5 (P<0.028) in patients compere with controls group.

In present study, when classifying patients with ischemic heart disease IHD into three types (stable Angina pectoris (SAP), Unstable angina pectoris (UAP) and Myocardial infarction (MI)), it was noted that the mean levels of CCL5 chemokine in the serum were uneven between the three types, as they were CCL5 serum level in UAP was slightly high than SAP and MI [(21.58 $\pm$ 5.03) and (19.53 $\pm$ 5.90) and (19.06 $\pm$ 6.06)]respectively. All the results showed an increase in CCL5 serum levels when compared with the control group (5.93 $\pm$ 1.70). statistically the differences was very highly significant for SAP and and no significant with UAP and MI that have [(P-value 0.001), (P-value 0.236) and (P-value 0.103)]-respectively. The results have been confirmed by previous studies (**Koper, et al, .2019**).

According to the study by Koper et al., 2009 mentioned above, CCL5 levels were higher in the MI and SAP groups compared to the control group, and statistically significant matching was found to be high for both MI and SAP (P > 0.001) with healthy patients.

Other studies have reported greater CCL5 levels in patients with MI compared to a healthy control group, but there was no difference in chemokine levels between patients with SAP and the healthy control group. (**Nomura, et al, .2003**) . Nomura, et al, . is It is consistent with our MI results, but inconsistent with our SAP group results.

On the other hand, **Cavusoglu, *et al*, . (2007)** show that a low CCL5 concentration was an independent predictor of myocardial infarction and cardiac death in men with known or suspected coronary artery disease. Our findings are contrary to the previous conclusion. These controversies may be attributed to the fact that CCL5 levels exhibit substantial ethnic diversity (**Virani et al, 2011**) or to the fact that our study group and Cavusoglu's study group were comprised of participants from different geographic regions. And distinct plasma/serum collecting samples.

Since atherosclerosis and thrombosis plague occur in the arteries, blood samples were taken from both sites of the same patient to compare the levels of chemokine in the vein sample and the levels of chemokine in the artery sample, Where the recent study showed patient with ischemic heart disease IHD have increase of mean chemokine CCL5 serum level in the artery sample compared to the mean $\pm$ SD in the vein [(23.30 $\pm$ 2.38) and (16.22 $\pm$ 7.73)] respectively.

Salim, *et al*, .(2011) refers to possibility secreted CCL5/RANTES by distinct cell types in the artery wall, which is consistent with our data indicating that CCL5/RANTES levels in tissues may differ from CCL5/RANTES levels in plasma. 3 Prior research shown a strong link between plasma and tissue CCL5 concentrations. (**Baer, et al, .2006**) However, there is still the possibility that despite low plasma levels, CCL5 tissue expression could be significant. Since the predominant action of CCL5 occurs in the vessel wall and not the circulation, this would be much more harmful. (**Salim, *et al*, .2011**).

4.3.3. chemokine CXCL9 (C-X-C ligand 9) :

CXCL9, also known as Monokine induced by gamma (MIG), is a chemokine that is induced by IFN- and has the capacity to amplify the IFN- gamma signal. (**Tokunaga, *et al*., 2018**). is an essential inflammatory chemokine in the recruitment of activated T-cells to sites of inflammation. MIG increases the polarization of Th1 and Th2 by attracting Th1 cells and preventing Th2

migration. (**hardison, *et al*., 2006)** . They primarily direct the recruitment of activated T cells to perform immunological tasks and prevent angiogenesis to some degree (**Metzemaekers, *et al* ., 2017)**.

Research on CXC chemokines suggests they are instrumental in the development of cardiovascular disease. As a result, they could be a target for many cardiovascular disease interventions. To wit: **(Lu, et al, . 2022).**

At the site of atherosclerosis, IFN-g may cause endothelial cells to produce CXCL9, so attracting and keeping activated T lymphocytes. Angina patients had increased levels of chemokines in their blood. (**de Oliveira, et al., 2009)**. According to research by Heller. et al. (2006), CXCL10 promotes atherosclerosis by recruiting activated T cells and inhibiting the aggregation of regulatory T cells (Tregs) at lesion sites. Since CXCL10's receptor, CXCR3, is also shared by CXCL9 and CXCL11, we postulate that these three chemokines may have similar functions. To wit: (**Andersen, et al, . 2019).**

Also some previous study shown high serum levels of IFN-g induced chemokine's CXCL9, 10, and 11 can be detected in human atheromases throughout all stages of plaque-development (**Zernecke, *et al* ., 2008**).

In the current study, results documented that the mean levels of chemokine's CXCL9 (pg\ml) among ischemic heart disease patients was higher than control group that have [(268.354 $\pm$ 249.981) and (25.554 $\pm$ 19.294)] respectively, statistically a differences was highly significant that have (P-value 0.001) and confirm the results reported by the previous study (**szentes, *et al*, .2018** and **zernecke, *et al*., 2008**(.

In present study, when classifying patients with ischemic heart disease IHD into three types (SAP, UAP and MI)), it was noted that the mean levels of CXCL9 chemokine in the serum were uneven between the three types, as they were CXCL9 serum level in MI was slightly high than SAP and high than USP [(285.55 $\pm$ 235.53) and (280.20 $\pm$ 274.14) and (216.97 $\pm$ 257.93)] respectively. All the results showed an increase of CXCL9 serum levels when compared with the control group (25.55 $\pm$ 19.29). statistically the differences was very highly significant for SAP and and no significant with UAP and MI that have [(P-value 0.001), (P-value 0.494) and (P-value 0.279)] respectively. The results have been confirmed by previous studies (**Fernandes, *et al*, . 2004**).

Fernandes, et al, .(2004) in their study observed significant higher mean level of chemokine CXCL9 in unstable angina (SAP) group when compared with control group, Unstable angina (UAP) patients, however, had CXCL9 and CXCR3 levels that were low compared to both the control group and those with stable angina. The authors postulated that the lower concentrations of these molecules in the peripheral circulation were due to their local release and strong absorption by circulating leukocytes moving to the region of active inflammation. We expected that serum increases in CXCR3 and related chemokine's would not be detected in blood samples taken more than 48 hours after the index consultation of the unstable patients . (**Fernandes, *et al*, . 2004**).

Since atherosclerosis and thrombosis plague occur in the arteries, blood samples were taken from both sites of the same patient to compare the levels of chemokine in the vein sample and the levels of chemokine in the artery sample, Where the recent study showed patient with ischemic heart disease IHD have increase of mean chemokine CXCL9 serum level in the artery compared to the mean $\pm$ SD in the vein [(284.578 $\pm$ 326.134) and (211.184 $\pm$ 233.913)] respectively. By studying human carotid plaque samples, **Segers, *et al*., (2011)** found a connection between elevated CXCL10 levels in the arteries and deteriorating plaques. Due to the fact that CXCL9 and CXCL11 bind to the same receptor, CXCR3, as CXCL10, we hypothesis that they serve the same function. According to a study (Lu, *et al*., 2022).

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